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A GTP Affinity Probe for Proteomics Highlights Flexibility in Purine Nucleotide Selectivity

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Supporting Information

ABSTRACT: GTP affinity probes are important tools for the study of GTP-binding proteins, and proteomic profiling is a powerful methodology well suited for the study of such a diverse class of proteins. Here, we synthesize and characterize a photoreactive GTP affinity probe that covalently photocross-links to protein targets and has an alkyne handle for click chemistry conjugation to reporter tags. The GTP-BP-yne probe facilitated identification of a variety of GTP-binding proteins by mass spectrometry, such as small GTPases and members of the GTP1/OBG family. Several ATP-binding proteins were also identified, highlighting variability in purine nucleotide selectivity of some proteins, and the probe was used to elucidate targets' relative nucleotide selectivities. The GTP-BP-yne probe will be a useful tool for the study of GTP-binding proteins, especially when targets of interest are not known a priori.

GTP-binding proteins constitute a vast, ubiquitous class of proteins essential for cell signaling, trafficking, cytoskeletal structure, nucelotide metabolism, and translation. Thus, GTP affinity probes are broadly applicable for the study of a variety of GTPases. Radioactive GTP analogs, such as [^{35}S]GTP and [a-^{32}P]GTP azidoaniline have facilitated characterization of GTPase signaling pathways.[1,2] However, radioactive or Western blots must be used to visualize predetermined protein targets of these probes. Mass spectrometry based protein profiling allows identification of a large number of unknown protein targets simultaneously. Although Kaneda et al. synthesized a GTP probe designed for proteomics,[3] it has not been characterized, and a commercially available GTP acyl phosphate relies on an active site lysine properly positioned for covalent linkage to its targets.[4] Here, we report the synthesis and characterization of a GTP affinity probe, GTP-BP-yne (**1**, Figure 1) for proteomic profiling. We used Multidimensional Protein Identification Technology (MudPIT)[5] to identify 33 proteins specifically labeled by **1** with high confidence, including members of several different classes of known GTP-binding proteins. Surprisingly, two of the highest confidence targets of **1** were ATP-binding proteins, one of which was not previously reported to bind GTP. Validation studies using in-gel fluorescence to visualize GTP-BP-yne–protein conjugates served two purposes: (1) they confirmed that targets discovered by MudPIT bind **1** specifically, and (2) they allowed direct analysis of relative

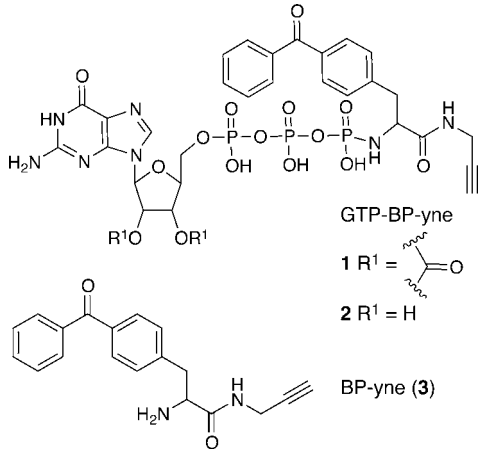

Figure 1. Structures of the GTP-BP-yne probes and control compound BP-yne.

GTP and ATP affinities of each target. Together, our results validate the utility of the GTP-BP-yne probe for use in mass spectrometry and gel based proteomics and for determining relative purine nucleotide selectivity of protein targets, while providing additional evidence[6,7] that purine nucleotide selectivity may not be as strict as it is often assumed to be.

Two structural features, a cross-linker moiety and handle for click chemistry[8,9] to append enrichment or fluorescent tags, must be added to GTP to make an affinity probe suitable for protemic profiling. Photoactivatable benzophenone was chosen as the cross-linker to allow control over the cross-linking timing, and an alkyne tag was used as the click chemistry handle. Structure–activity relationships of previously reported GTP analogs showed that modification of the gamma-phosphate and 2′ and 3′ hydroxyl groups minimally impacts binding to GTP-binding proteins.[3,10−15] We appended both the photocross-linking and alkyne moieties to the gamma phosphate of GTP. CDI mediated coupling of **3** to GTP yielded the 2′,3′-carbonate, **1** (Scheme S1). When CDI was limiting and the reaction monitored to ensure the cyclic carbonate was not formed before **3** was added, the 2′,3′-diol (**2**) was formed in low yield (Materials and Methods, SI). The 2′,3′-carbonate is a minor modification relative to the bulky fluorescent groups appended to the hydroxyl groups of commercially available GTP analogs[13,15] and commonly used

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for the study of nucleotide-protein interactions. And a comparison of lysate labeling by **1** and **2** showed that both probes have very similar profiles (Figure S1). Thus, the synthesis of the higher yielding probe, **1**, was scaled up for use in mass spectrometry based proteomic studies.

To determine the protein targets of **1**, MudPIT[5] experiments were carried out with HEK 293T cells. Briefly, cell lysates were fractionated into soluble and insoluble fractions, and endogenous nucleotides were removed by desalting. Proteomes were labeled with 30 μM **1** in the presence of 60 mM $MgCl_2$, then irradiated with 365 nm light, followed by addition of a biotin tag via click chemistry. To exclude nonspecific targets of **1**, two control samples were generated in each sample set: (1) excess GTP was added to compete away specific GTP-BP-yne–protein interactions and (2) BP-yne (**3**, Figure 1) was added instead of **1** to identify any targets that were labeled due to interaction with the non-nucleotide portion of the probe or due merely to high abundance of the target. The labeled proteomes were reduced, alkylated, enriched for labeled targets using streptavidin beads, and trypsinized.[16] The tryptic peptides were analyzed by MudPIT and protein targets were identified using ProLuCID[17] and quantified by spectral counting.[18] True target proteins were identified as those that met threshold criteria in each of three replicate data sets. First, targets with fewer than 3 spectral counts were eliminated. Next, the spectral counts of all targets in each sample were normalized based on the total number of spectral counts of each sample. The normalized spectral counts were used to calculate spectral count ratios of samples labeled with GTP-BP-yne to the control samples, and only targets with ratios of 2 or greater were considered to be true hits. Ratios from all three data sets were then averaged.

Thirty-three proteins met the above criteria in all three data sets (Tables 1, S1), and 77 proteins met the criteria in two out of three data sets (Table S2), including many known GTP-binding proteins. Thus, **1** works as designed to bind, label, and enrich GTP-binding proteins in mass spectrometry based proteomics. Interestingly, the GTP-binding hit proteins are members of diverse GTP-binding protein classes (Figure 2), including small Ras-related GTPases (e.g., Rab10), heterotrimeric G alpha (e.g., Ga_{i2}), unusual GPN-loop GTPases (e.g., GPN1), translation elongation factors (e.g., EEF1A1), and some proteins of unknown function that have been annotated as GTP-binding based on their sequence (e.g., GTPBP6 of the MMR1/HSR1 family), thus demonstrating the utility of **1** for studying a variety of GTP–protein interactions.

Furthermore, the target proteins described here were identified using one set of conditions and thereby represent a minimalist set of possible hits. It is possible that varying parameters, such as divalent cation concentration, could result in even greater coverage of the GTP-binding proteome.

Table 1. Top Five Targets of GTP-BP-yne (1)

protein	average spectral counts	1 vs 1 + GTP	1 vs 3	NT ligand(s)
BCS1L	207.08	14.35	33.51	ATP
GNL3	72.95	5.45	7.98	GTP
CSNK2A2	45.33	27.95	3.06	ATP, GTP
ATL3	43.95	Undef[a]	23.72	GTP
OPA1	40.71	3.18	3.40	GTP

[a]Undef = Undefined. The spectral counts for the control sample was equal to zero in all three replicates.

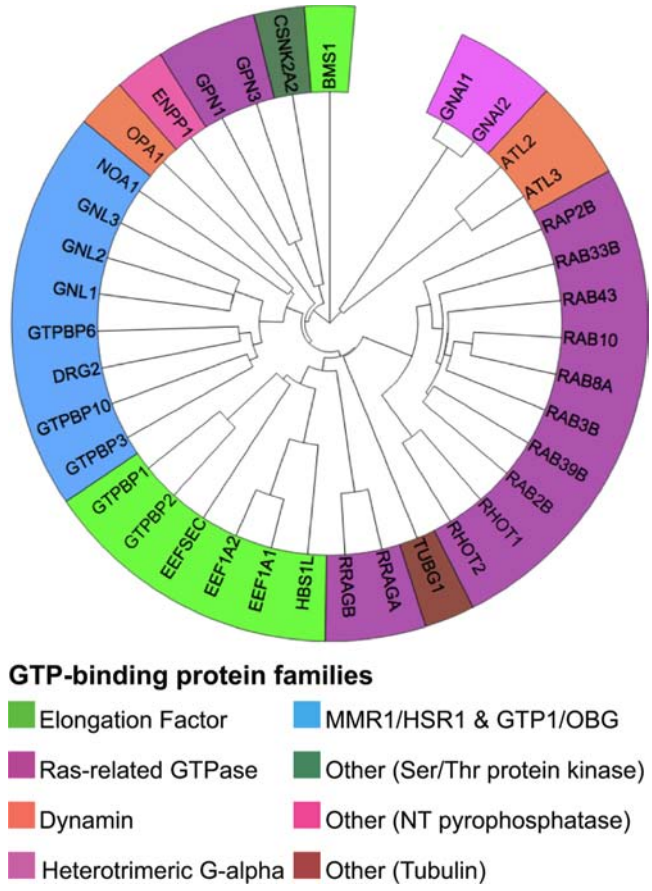

Figure 2. Dendrogram of the GTP-binding protein targets of GTP-BP-yne (**1**).

To confirm that **1** specifically binds target proteins' GTP binding sites, we transiently transfected five targets identified by mass spectrometry in HEK 293T cells and labeled the resulting lysates with **1**. Azide-Rhodamine was conjugated to the GTP-BP-yne–protein complexes via click chemistry, and the samples were separated by SDS-PAGE and visualized with a fluorescence scanner. This method allowed direct visualization of the labeled targets in the presence and absence of excess nucleotide competitors and immediate qualitative comparison of the targets' relative affinities for GTP and ATP. Atlastin-3 (ATL3, Table 1) and Mitochondrial Rho GTPase 2 (RHOT2, Table S1), two GTPase targets identified in the insoluble fraction, were confirmed using this method (Figure 3A). Binding of **1** to both GTPases was competed by excess GTP but not noticeably changed in the presence of excess ATP, as expected. Casein kinase II alpha′ (CSNK2A2, Table 1) utilizes both GTP and ATP as phosphate donors,[19] and labeling by **1** was eliminated by excess GTP and ATP (Figure 3C).

While **1** labeled many known GTP-binding proteins as expected, several hits bind ATP, nucleic acids, or other proteins that bind purine nucleotides but are not known to bind GTP. This target profile is similar to the profile of the ATP acyl phosphate probe made by Patricelli et al. that labels targets via nucleophilic attack by an active site lysine.[4] Most of the ATP probe targets are ATP-binding proteins, and other targets include proteins that bind nucleic acids, NAD, and FAD. Only 1.5% of the ATP probe targets bind GTP, in contrast with the 26% of the GTP-BP-yne targets that bind ATP, but the stricter

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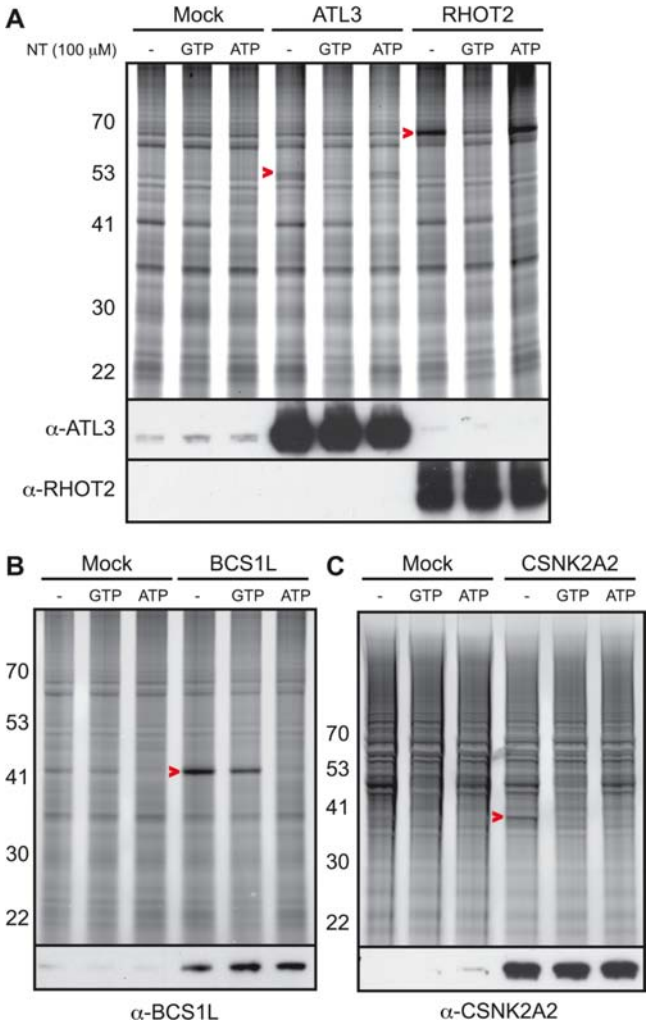

Figure 3. Validation and relative purine nucleotide affinity of selected targets by overexpression, labeling with **1**, conjugation to azide-rhodamine, and in-gel fluorescence. Western blots shown below each gel confirmed target overexpression. Excess GTP or ATP was added to assess nucleotide preference. Arrows indicate proteins of interest. (A, B) Insoluble fraction. (C) Soluble fraction.

nucleotide selectivity observed with the ATP probe may be due to its unique labeling mechanism and structure.

Targets of **1** that are not annotated as GTP-binding proteins include the related kinases Src and Lyn are high confidence targets of **1** (Table S1), and Yes kinase was also identified by **1** (Table S2). Src kinase has been reported to use GTP as a phosphate donor *in vitro*,[20] but GTP binding by family members Lyn and Yes has not been tested to our knowledge. RFC1 and 5, two ATPase subunits in the large DNA replication complex, are targets of **1** (Tables S1, S2), suggesting that the replication factor complex may hydrolyze GTP and ATP. The gene product of NUDT2, Ap_4A hydrolase, primarily hydrolyzes diadenosine tetraphosphate as well as other dinucleoside phosphates,[21] and our labeling studies indicate that it binds GTP and ATP with similar affinities (Figure S2). We also identified two high confidence targets that are not known to bind any nucleotides, COPB2 and AP3B1 (Table S1), members of the COPI coat complex and clathrin complex, respectively. COPB2 binds Arf·GTP, and the crystal structure of the complex shows that the gamma phosphate of the Arf-bound GTP is close to COPB2.[22] It is possible that cross-linking can occur to a bystander protein within a protein−protein complex when the GTP binding pocket orients the benzophenone in close proximity to the binding partner. Thus, **1** may also be useful for the study of proteins that bind GTP-binding proteins even if they do not bind a nucleotide themselves. And BCS1L, the hit with the highest spectral counts (Table 1), is a unique AAA ATPase localized to the inner mitochondrial membrane that is not known to bind GTP.[23]

BCS1L is essential for formation of respiratory complex III in mitochondira, and certain mutations cause the fatal GRACILE syndrome.[24] Sequence analysis puts BCS1L in a subclass of its own, distinct from other AAA ATPases,[25] and it is required for maturation of the Fe−S cluster containing Rieske protein in *Saccharomyces cerevisiae*.[23] Labeling of BCS1L by **1** was confirmed by the appearance of a dark fluorescent band at 48 kDa, which is even observed in mock-transfected samples (Figure 3B), consistent with the high spectral counts observed by mass spectrometry. While GTP competition diminished BCS1L labeling, ATP competition completely abolished it, showing that ATP is indeed preferred over GTP but may not be the only physiological nucleotide ligand, especially since both ATP and GTP are known to be required for maturation of iron−sulfur cluster proteins in mitochondria via unknown mechanisms. Although we could not detect GTPase (or ATPase) activity with purified BSC1L in an inorganic phosphate assay, it is possible that other cofactors or membrane components may be required for BCS1L activity.

Taken together, our results show that GTP-BP-yne, **1**, functions as the first GTP affinity probe demonstrated to be compatible with proteomics, and it facilitated the identification of proteins previously unknown to bind GTP, such as the ATPase BCS1L, and to evaluate the purine nucleotide selectivities of the protein targets.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental tables and figures, materials and methods, and synthetic scheme. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): HR is a consultant to Activx Biosciences and a scientific co-founder of Receptos.

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